Cryobiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing ☆

Ki-Jung Kim [a], Yong-An Lee [a,1], Bang-Jin Kim [a], Yong-Hee Kim [a], Byung-Gak Kim [b], Hyun-Gu Kang [a], Sang-Eun Jung [a], Sun-Ho Choi [c], Jonathan A. Schmidt [d], Buom-Yong Ryu [a,*]

[a] Department of Animal Science and Technology, Chung-Ang University, Ansung, Gyeonggi-Do 456-756, Republic of Korea
[b] Obstetrics, Gynecology & Reproductive Biology, Michigan State University, USA
[c] National Institute of Animal Science, RDA, Cheonan 331-801, Republic of Korea
[d] Department of Science, Spokane Community College, 1810 N Greene St., Spokane, WA 99217-5399, USA

ARTICLE INFO

Article history:
Received 20 November 2014
Accepted 23 February 2015
Available online xxxx

Keywords:
Bovine
Cryopreservation
Slow freezing
Spermatogonial stem cell
Trehalose

ABSTRACT

Development of techniques for the preservation of mammalian spermatogonial stem cells (SSCs) is a critical step in commercial application of SSC based technologies, including species preservation, amplification of agriculturally valuable germ lines, and human fertility preservations. The objective of this study was to develop an efficient cryopreservation protocol for preservation of bovine SSCs using a slow freezing technique. To maximize the efficiency of SSC cryopreservation, the effects of various methods (tissue vs. cell freezing) and cryoprotective agents (trehalose, sucrose, and polyethylene glycol [PEG]) were tested. Following thawing, cells were enriched for undifferentiated spermatogonia by differential plating and evaluated for recovery rate, proliferation capacity, and apoptosis. Additionally, putative stem cell activity was assessed using SSC xenotransplantation. The recovery rate, and proliferation capacity of undifferentiated spermatogonia were significantly greater for germ cells frozen using tissue freezing methods compared to cell freezing methods. Cryopreservation in the presence of 200 mM trehalose resulted in significantly greater recovery rate, proliferation capacity, and apoptosis of germ cells compared to control. Furthermore, cryopreservation using the tissue freezing method in the presence of 200 mM trehalose resulted in the production of colonies of donor-derived germ cells after xenotransplantation into recipient mouse testes, indicating putative stem cell function. Collectively, these data indicate that cryopreservation using tissue freezing methods in the presence of 200 mM trehalose is an efficient cryopreservation protocol for bovine SSCs.

Introduction

Spermatogenesis is a complex process initiated by spermatogonial stem cells (SSCs) that have the ability to differentiate into mature spermatozoa or to self-renew to maintain the SSC population and long-term fertility [5,7,33]. Because SSCs are the only adult stem cell capable of contributing genetic material to the next generation, commercial/clinical manipulation of these cells will lead to the development of techniques to amplify valuable mammalian agricultural germ lines, preserve the germ line of endangered species and treat human infertility caused by prepubertal cancer treatment. Unfortunately, SSCs are extremely rare, comprising only 0.03% and 0.2% of adult mouse and rat testis cells respectively [34,35]. Therefore to develop germ line preservation techniques, methods for isolation, enrichment, culture, transplantation, characterization, and preservation of the SSC must be developed [1,3,11,19,21,24].

Previous studies have demonstrated the potential for long-term culture and cryopreservation of rodent SSCs [2,28,29,31]; however, there are currently no described methods for long-term culture of bovine SSCs. Some studies have reported cryopreservation methods for undifferentiated spermatogonia of livestock, including bulls [6,15,22], but analysis of different cryopreservation techniques for bovine germ cells is lacking. Development of highly effective cryopreservation techniques is important for a variety of reasons. These include being a more efficient long-term preservation technique

☆ *Statement of funding:* This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (H12C0055) and the Next-Generation BioGreen 21 Program (Grant PJ011347).

* Corresponding author. Fax: +82 31 676 0062.
E-mail address: byryu@cau.ac.kr (B.-Y. Ryu).
[1] Current address: Department of Chemistry, National University of Singapore, Republic of Singapore.

http://dx.doi.org/10.1016/j.cryobiol.2015.02.007
0011-2240/

than culture because cryopreservation reduces contamination, avoids the effects of aging, diminishes transformation in finite lines, and minimizes genetic change in continuous lines [38].

Over the past decade, several studies have demonstrated that cryopreservation of undifferentiated germ cells using slow freezing methods is more effective than either fast freezing methods or vitrification [16,39]. The effectiveness of slow freezing is most likely due to decreased cell damage caused by intracellular ice formation or inappropriate dehydration [27,38]. In addition to freezing rate, cryo-damage can also be minimized by utilizing cryoprotective agents [13] such as trehalose, a non-reducing disaccharide of glucose, which prevents intracellular ice formation and desiccation [4,8]. Sugars, including trehalose and sucrose, increase the efficiency of cryopreservation of many cell types, including embryos, fibroblasts, hematopoietic stem cells, and germ cells [9,16,22,26,32]. Furthermore, the inclusion of trehalose and other sugars in cryopreservation media can significantly increase the recovery, viability, proliferation capacity, and colonization efficiency of mammalian undifferentiated spermatogonia [16,22,24]. In addition to sugars, inclusion of polyethylene glycol (PEG) in cryopreservation media for undifferentiated spermatogonia increases cell viability, recover, culture potential and colonization efficiency presumably by protecting the plasma membrane rather than preventing formation of intracellular ice [23].

The objective of the present study was to develop an effective cryopreservation protocol for bovine SSCs by testing different freezing methods (tissue vs. cell freezing) and various cryoprotective agents (including trehalose, sucrose, and PEG) using slow freezing protocols. Determination of the most effective method of cryopreservation was conducted by evaluating the post-thaw recovery rate, proliferation capacity, apoptosis percentage, and functionality of putative SSCs. Results of this study will be integral for the continued refinement of techniques to manipulate mammalian undifferentiated spermatogonia including spermatogonial stem cells.

Materials and methods

Acquisition of donor testes

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). All animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research* and the *Guide for Care and Use of Laboratory Animals*. Donor testes were obtained from 10- to 14-week-old prepubertal Holstein–Friesian bull calves by standard castration procedures. After castration, testes were placed in Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen, Grand Island, NY, USA) and stored on ice for 1–2 h during transport. Under sterile conditions, the tunica vaginalis and tunica albuginea were removed, and the testicular parenchyma was washed in DPBS.

Cryopreservation

To optimize cryopreservation of bovine undifferentiated spermatogonia, two different cryopreservation protocols (tissue freezing or cell freezing) and various cryoprotective agents (trehalose, sucrose, and PEG) were evaluated as described previously [16,23,24] with minor modifications. Briefly, 0.5 mL of stock cryopreservation media [20% v/v dimethylsulfoxide (DMSO), 60% v/v PBS, and 20% v/v heat-inactivated fetal bovine serum (FBS; Hyclone, Thermo Scientific, Logan, UT)] was added drop-by-drop to tissues and cells previously suspended in 0.5 mL of PBS containing cryoprotective agents [none, 140 mM or 400 mM trehalose, 140 mM or 400 mM sucrose, or 5% PEG (molecular weight, MW 1000)] to a final volume of 1 mL and placed into 1.8 mL cryovials [Corning, Midland, MI, USA; final cryoprotective agent concentration were 70 mM or 200 mM trehalsoe, 70 mM or 200 mM sucrose, or 2.5% PEG (MW 1000) in PBS containing 10% v/v DMSO and 10% v/v FBS respectively]. Prior to freezing, the cryovials were incubated on ice for at least 15 min with occasional agitation to permit complete cryoprotectant diffusion into the testis sample [17]. Following incubation, vials were placed overnight into a freezing container (Nalgene, Rochester, NY, USA) containing 100% isopropyl alcohol that provided a ~1 °C/min cooling rate to −80 °C. The following morning, vials were placed into liquid nitrogen. Samples were thawed after one month by incubation in a 37 °C water bath for 3.5 min (tissue freezing) and/or 2.5 min (cell freezing). To minimize donor variation, one testis from each calf was used for the tissue freezing methods and the contralateral testis was used for the cell freezing methods. To prepare tissue for the tissue freezing method, testis tissue was dissected into 2–3 mm^3 pieces and 1 g of testis tissue was frozen per cryovial. After thawing, testis tissue was digested into single-cell suspension. To prepare cells for the cell freezing method, the contralateral testis was chopped with forceps and scissors and digested into a single-cell suspension. Cells were frozen at a concentration of $\sim 100 \times 10^6$ live cells per vial.

Preparation of single-cell suspensions

To create single cell suspensions, a two-step enzymatic isolation procedure was used as previously described [12,14,20] with minor modifications. Briefly, following chopping with scissors, the isolated testis tissues were washed three times with Dulbecco's modified Eagle medium (DMEM; Invitrogen) at 10 min intervals. The tissues were then digested with colla genase type IV (2 mg/mL) in DPBS at 37 °C for 40 min with occasional agitation. After digestion, the resulting testis fragments were further digested with collagenase (2 mg/mL) and hyaluronidase (2 mg/mL) in DPBS at 37 °C for 15 min. Digested testis fragments were washed three times with DPBS and incubated in a 4:1 solution of 0.25% trypsin in 1 mM EDTA (Invitrogen) and DNase I (7 mg/mL; Roche, Basel, Switzerland) in DPBS at 37 °C for 10 min. Trypsin was inactivated by the ad dition of 10% FBS. The resulting cell suspension was then filtered through a nylon mesh cell strainer (70 μm pore size; BD biosciences, San Jose, CA, USA) and re-suspended in basic medium (DMEM containing 10% FBS, 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol, 100 units [U]/mL penicillin, and 100 μg/mL streptomycin [Invitrogen]). The cell suspension was washed in basic medium and centrifuged two times at 600 × *g* at 4 °C for 7 min. After centrifugation, the cell pellet was re-suspended in basic medium.

Selection and Enrichment of Undifferentiated Spermatogonia

To enrich undifferentiated spermatogonia prior to freezing (cell freezing method) or after thawing (tissue freezing method) digested cells were subjected to discontinuous Percoll density gradient centrifugation followed by differential plating. First, to isolate testis cells from erythrocytes and cellular debris, discontinuous Percoll density gradient centrifugation was performed as previously described [10,20] with minor modifications. Briefly, two Percoll densities (2 mL each), 20% and 40%, were layered in a 15 mL centrifuge tube (BD Biosciences) and single-cell suspensions (2 mL containing 5×10^6 cell/mL) were loaded on top of layered 20% and 40% Percoll solutions and centrifuged at 600 × *g* at 4 °C for 10 min. Cells were harvested from the interface between the 20% and 40% Percoll layers and washed and centrifuged 600 × *g* at 4 °C for 7 min two times with basic medium.

Please cite this article in press as: K.-J. Kim et al., Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing, Cryobiology (2015), http://dx.doi.org/10.1016/j.cryobiol.2015.02.007